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13. ABSTRACT *(Maximum 200 words)*

The use of quantitative polymerase chain reaction (QPCR) for assessing PCP infection and its response to treatment was studied in rats. Groups of 8 rats were immunosuppressed with steroids for 3-12 weeks. Controls were maintained untreated for the same periods. Three groups of rats were treated with pentamadine, and three additional groups with trimethoprim and sulfamethoxazole while immunosuppressed. Three groups of rats were tapered from steroids. At various times during suppression the rats were sacrificed. At necropsy the lungs were lavaged and the BAL fluid assayed by cyst counting and QPCR using ribosomal DNA to quantitate PCP load in using a rat globin internal standard. Lung homogenates were similarly assayed. An increase in the QPCR signal was seen throughout immunosuppression, followed by a slow decrease upon withdrawal of steroids or a faster decrease with drug treatment. Results from QPCR of lung and BAL fluid were strongly correlated with each other and with cyst counts. Results warrant investigation of QPCR for assessing treatment results in human PCP infection.

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Timothy J. O'Leary 25 March 93

Principal Investigator's Signature Date

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FINAL REPORT

Polymerase Chain Reaction Assays for Diagnosis of *Pneumocystis Carinii* Infections

PRINCIPAL INVESTIGATORS: Timothy J. O'Leary, M.D., Ph.D.
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OBJECTIVES

The objectives of this study were to develop polymerase chain reaction assays for the identification and quantitative of *Pneumocystis carinii* organisms in the lungs, solid organs, bronchioloalveolar lavage fluids and blood of rats and humans.

All objectives for this study have been met, except for validation of a quantitative blood assay.

INTRODUCTION

Although drugs for treating *Pneumocystis carinii* infections are available, this pathogen nevertheless remains a major cause of death in immunosuppressed patients, particularly those harboring infection by the human immunodeficiency (HIV) virus. Currently, diagnosis depends upon correct interpretation of a constellation of clinical signs and symptoms, radiographic findings, and laboratory analysis of sputum, bronchioloalveolar lavage and lung biopsy. Assessing treatment efficacy is difficult, because patients may harbor other organisms, such as cytomegalovirus, which result in similar organ system effects, because radiographic findings often lag behind effective treatment by days to weeks, because routine histopathologic examination of organisms may fail to differentiate between viable and nonviable cysts, and because visualization of trophozoites is often difficult using routine histopathologic methods.

Quantitative polymerase chain reaction (QPCR) is potentially an attractive alternative to traditional histopathologic methods because it is quantitative, sensitive to viability of the organism, and potentially amenable to detection not only in bronchioloalveolar lavage fluid, but also in sputum and blood, both of which are much easier to obtain. The objective of this investigation was to determine if, in fact QPCR could be used to identify

Pneumocystis carinii infection and used to assess the efficacy of treatment in a previously investigated rat model.

EXPERIMENTAL DESIGN AND METHODS

Sasco Sprague-Dawley (SSD+) (O'Fallon colony, Room OM1, Omaha, Nebraska) were used for this investigation. The rats were maintained in barrier-isolated conditions and fed a normal diet. Some of the rates were maintained without immunosuppression, while others were immunosuppressed with methyprenisolone, 0.2 mg/week. After six weeks of this treatment, all rats have established a clinically significant level of immunosuppression and significant *Pneumocystis carinii* infections. After six weeks of initial immunosuppression, three groups of 8 immunosuppressed rats were treated with the anti-*P. carinii* drugs pentamadine and three additional groups of 8 rats were treated with trimethoprim-sulfamethoxazole while remaining immunosuppressed by methylprednisolone. The numbers of rats in each group and their treatment is summarized in Table 1. Groups of rats were sacrificed at time zero, and then at regular intervals for the duration of the study, then necropsied. A number of tissues, including blood, lung, liver, spleen, bone marrow, testes, and kidney were taken at necropsy and fixed in 95% ethanol. Prior to fixation, the lung was lavaged with saline. A portion of the lavage fluid was retained for cyst counting, and the remainder was centrifuged and separated into two fractions, one of which contained cell pellet and the other of which consisted of supernatant.

The pulmonary tissue from each animal was minced and prepared for PCR, the detailed protocol for which appears in Appendix 1. After preliminary experiments demonstrated more reproducible quantitative PCR results from the cell pellet fraction of the bronchioloalveolar lavage (BAL) fluid, further quantitative PCR measurements for each animal were also carried out as described in Appendix 1.

Data analysis was carried out using the Systat Computer Program (Systat Inc. Evanston, IL). Analysis of variance was followed by post test comparison using the Tukey HSD method. Linear regression analyses were also carried out.

RESULTS

The results of lung and BAL PCR experiments for each animal are given in Appendix 2.

Lung

Results of the initial immunosuppression experiments

are shown in Figure 1A. There is a substantial increase in the mean quantitative PCR signals for pulmonary tissue in the interval between 0 and three weeks, which increases only somewhat in the ensuing nine weeks. Individual rats differed substantially in their responses to the immunosuppression, however. Among forty-four nonimmunosuppressed rats whose lungs were available for assay, one had a QPCR measurement of 28, for example, while one rat immunosuppressed for three weeks at a QPCR of 15, and one immunosuppressed for 9 weeks had a QPCR of 22. Whether this represents inter-individual variation in the response to immunosuppression or limitations in the lavage/assay techniques used in this series of experiments is unclear. Nevertheless, a statistically significant difference in the QPCR result is seen by week 9.

The effects of withdrawing steroids is illustrated in Figure 2A. Rats withdrawn from steroids show a decreased QPCR for PCP which approaches pre-immunosuppression levels by the end of the experiment.

The effects of treatment with trimethoprim-sulfamethoxasole (TMP) are seen in Figure 3A. Treatment with trimethoprim-sulfamelthoxazole causes a rapid decrease in the PCP QPCR result; by the first time point, the results are not significantly different from those of control animals which had never been immunosuppressed.

The effects of treatment with pentamadine are illustrated in Figure 4A. The treatment appears to be somewhat less effective, as measured by the QPCR method, than atrimelthoprim-sulfamethoxazolae; the differences are not statistically significant, however.

BAL Fluid

Results of the initial immunosuppression experiments are shown in Figure 1B. There is a substantial increase in the mean quantitative PCR signals for BAL fluid in the interval between 0 and three weeks, which increases only somewhat in the ensuing nine weeks. Individual rats differed substantially in their responses to the immunosuppression, however. Among fifty nonimmunosuppressed rats whose BAL fluid was available for assay, one had a QPCR measurement of 10, for example, while one rat immunosuppressed for three weeks at a QPCR of 0, and one immunosuppressed for 6 weeks had a QPCR of 7. Whether this represents inter-individual variation in the response to immunosuppression or limitations in the lavage/assay techniques used in this series of experiments is unclear. Nevertheless, a statistically significant difference in the QPCR results is seen by week 7.5 and persists through the twelve week sample. Statistically significant differences in lung are seen by week 9.

The effects of withdrawing steroids is illustrated in Figure 2B. Rats withdrawn from steroids show a decreased QPCR for PCP which approaches pre-immunosuppression levels by the end of the experiment.

The effects of treatment with trimethoprim-sulfamethoxasole (TMP) ares seen in Figure 3B. Treatment with trimethoprim-sulfamelthoxazole causes a rapid decrease in the PCP QPCR result; by the second time point, the results are not significantly different from those of control animals which had never been immunosuppressed.

The effects of treatment with pentamadine are illustrated in Figure 4B. The treatment appears to be somewhat less effective, as measured by the QPCR method, than atrimelthoprim-sulfamethoxazolae; the differences are not statistically significant, however.

Results of correlation analyses between BAL QPCR Results and lung QPCR results are shown in Figure 5. The relationship between the values may be summarized by the equation

$$\text{BAL QPCR} = (0.462 * \text{Lung QPCR}) + 21.6251.$$

The Pearson correlation coefficient for the relationship is 0.435, and the correlation is significant at $p < 0.001$. Nevertheless, as the scatter plot in Figure 5 indicates, there is substantial scatter in the data. This may reflect the sampling difficulties associated with both lung PCR and BAL collection in rats, as noted above.

Blood

Preliminary experiments were performed in which rat *Pneumocystis carinii* organisms were quantitated by sorting on an Ortho Cytofluorograph flow cytometer, then doped into human blood. The protocol found in Appendix 3 was then used to isolate DNA from the samples. These experiments demonstrated that we can detect and quantitate organisms at the level of 10 cysts/50 microliters (200 cysts/ml). This may be a sufficiently sensitive detection system to allow detection of *Pneumocystis carinii* infection via a blood test.

Experiments on blood from the animals whose results are discussed above were considered unsatisfactory, possibly as a result of difficulties associated with fixation of the samples by ethanol. We are currently investigating the potential usefulness of the blood assay by performing serial bleeding experiments on 10 animals who were first bled (control) then immunosuppressed with methylprednisolone, as described above, innoculated with *Pneumocystis carinii*, bled soon after inoculation and then periodically for several weeks thereafter. By bleeding after inoculation we will be able to determine

whether the blood assay works, and by bleeding and assaying for several weeks after this inoculation we should be able to determine if the blood-based PCR assay is useful for following infection after the initial innoculum has been scavenged by the reticuloendothelial system.

DISCUSSION

The experimental results presented above suggest that QPCR is an effective approach by which to identify clinically significant *Pneumocystis* infections in the rat, and to follow treatment. Neverthtless, the degree of rat to rat variation is high. Only by serially following infection in single individuals using QPCR will the utility of this technique be clearly demonstrated or disproved.

We can serially follow results of blood assays in the rat model, but cannot perform sputum or BAL examinations in this system. A decision on the next course of action awaits results of the experiments on quantiation of organisms in blood.

PUBLICATIONS

Two publications are in preparation. Copies of the submitted manuscripts will be forwarded to the Army Medical Research and Development Command when available.

SUPPLEMENTAL REPORT

After the final results on the blood assays have been obtained, a supplement to this report will be issued and forwarded for review to the Army Medical Research and Development Command.

Table 1 - Group (as used in appendix), Rat numbers, Treatment regimens and sampling times for animals used in this study

Group	Rats	Regimen	Time point
[Immunosuppressed, but treated after 6 weeks with trimethoprim-sulfamethoxazole (TMP-SMX)]			
13	1293-1300	Immunosuppressed but treated with TMP-SMX for 10 days at a dosage of 250 mg/kg per day.	7.5 weeks
4	1301 1303-1309	Immunosuppressed but treated with TMP-SMX for 21 days at a dosage of 250 mg/kg per day.	9 weeks
17	1310, 1311 1313 1315-1318	Immunosuppressed for 6 weeks, then treated with TMP-SMX for 6 weeks trimethoprim-sulfamethoxazole for 6 weeks	12 weeks
[Immunosuppressed, but treated after 6 weeks with pentamadine]			
14	1319-1327	Immunosuppressed by treated with pentamadine at dosage of 10 mg/kg 3 times per week for a total of 5 doses	7.5 weeks
5	1328-1332 1334-1336	Immunosuppressed, but treated with pentamadine for 21 days at a dosage of 10 mg/kg three times per week for a total of 7 doses.	9 weeks
18	1338-1344	Immunosuppressed but treated with pentamadine at a dosage of 10 mg/kg 3 times per week for 6 weeks	12 weeks
[Immunosuppressed with steroids, then tapered off]			
3	1259 1261-1267	Received full steroid injections for 6 wks, then tapered to 0.1 mg/wk for one week and to 0.05 mg/wk for one week, then sacrificed at week 9	9 weeks
16	1268-1274	Immunosuppressed for 6 weeks, then tapered, then taken off steroids altogether.	12 weeks
20	1275-1282	Full steroid injections for 6 weeks, tapered for 3 weeks,	15 weeks

		taken off steroids altogether for 5 weeks	
22	1283-1292	Immunosuppressed for 6 weeks, tapered for 3 weeks, off steroids for 9 weeks	18 weeks
[Non-immunosuppressed controls]			
11	1175-1182	Non-immunosuppressed controls	3 weeks
6	1183-1190	Non-suppressed control, 6 weeks	6 weeks
8	1191-1198	Non-suppressed, 7.5 weeks	7.5 weeks
1	1199-1206	Non-immunosuppressed rats (negative controls)	9 weeks
19	1207-1210	Never immunosuppressed, kept in barrier isolation for 15 weeks	15 weeks
21	1211-1214	No immunosuppression for 18 wks	18 weeks
[Immunosuppressed by injecting with methylprednisolone, 0.2 mg/wk]			
12	1215-1222	Steroid suppressed, 3 weeks	3 weeks
7	1223-1230	Steroid suppressed, 6 weeks	6 weeks
9	1231-1238	Steroid suppressed, 7.5 weeks	7.5 weeks
2	1241-1247 1249	Corticosteroid injections for 9 weeks (0.2 mg/wk)	9 weeks
15	1239, 1240 1250 1256-1258	Immunosuppressed for 12 weeks. These rats are barely alive.	12 weeks

Table 2 - p Values for Differences in the Means Between Groups of Rats, as Computed by Tukey's HSD Method for QPCR Results Based on Pulmonary Tissue

	Control	Ster 9 wk	Ster 12 wk	TMP 1	TMP 2	TMP 3	Pent 1	Pent2	Pent 3
Control	1.000								
Ster - 9 wk	<u>0.000</u>	1.000							
Ster - 12 wk	<u>0.020</u>	0.999	1.000						
TMP 1	1.000	<u>0.033</u>	0.671	1.000					
TMP 2	1.000	<u>0.004</u>	0.316	1.000	1.000				
TMP 3	1.000	<u>0.002</u>	0.193	1.000	1.000	1.000			
Pent 1	0.447	0.465	0.998	0.999	0.952	0.853	1.000		
Pent 2	0.994	0.052	0.790	1.000	1.000	1.000	1.000	1.000	
Pent 3	1.000	<u>0.011</u>	0.486	0.903	1.000	1.000	1.000	1.000	1.000

Results which are statistically significant at the level of $p < 0.05$ are underlined.

Table 3 - p Values for Differences in the Means Between Groups of Rats, as Computed by Tukey's HSD Method

	Control	Ster 9 wk	Ster 12 wk	TMP 1	TMP 2	TMP 3	Pent 1	Pent2	Pent 3
Control	1.000								
Ster - 9 wk	<u>0.000</u>	1.000							
Ster - 12 wk	<u>0.003</u>	0.747	1.000						
TMP 1	<u>0.000</u>	<u>0.033</u>	0.998	1.000					
TMP 2	1.000	<u>0.000</u>	0.271	<u>0.003</u>	1.000				
TMP 3	1.000	<u>0.000</u>	0.098	<u>0.001</u>	1.000	1.000			
Pent 1	<u>0.000</u>	0.618	1.000	0.995	0.175	0.052	1.000		
Pent 2	0.833	<u>0.003</u>	0.867	0.075	1.000	0.998	0.803	1.000	
Pent 3	<u>0.022</u>	0.286	1.000	0.903	0.614	0.299	1.000	0.991	1.000

Results which are statistically significant at the level of $p < 0.05$ are underlined.

FIGURE LEGENDS

Figure 1A Average lung quantitative PCR (QPCR) results for non-immunosuppressed rats and rats which have been immunosuppressed with methylprednisolone for 3, 7.5, 9 and 12 weeks.

Figure 1B Average BAL QPCR results for non-immunosuppressed rats and rats which have been immunosuppressed with methylprednisolone for 3, 6, 7.5, 9 and 12 weeks.

Figure 2A Average lung QPCR results for rats which have been immunosuppressed for 9 weeks with methylprednisolone, and for rats which have been immunosuppressed for 6 weeks, tapered off steroids for the following three weeks, and left off steroids.

Figure 2B Average BAL QPCR results for rats which have been immunosuppressed for 9 weeks with methylprednisolone, and for rats which have been immunosuppressed for 6 weeks, tapered off steroids for the following three weeks, and left off steroids.

Figure 3A Average lung QPCR results for rats which have been immunosuppressed for 9 weeks with methylprednisolone, and for rats which have been immunosuppressed for 6 weeks, then treated with trimethoprim-sulfamethoxazole (TMP-SMX) while remaining immunosuppressed. Groups labeled TMP1, TMP2 and TMP 3 have been treated with TMP-SMX for progressively longer periods.

Figure 3B Average BAL QPCR results for rats which have been immunosuppressed for 9 weeks with methylprednisolone, and for rats which have been immunosuppressed for 6 weeks, then treated with trimethoprim-sulfamethoxazole (TMP-SMX) while remaining immunosuppressed. Groups labeled TMP1, TMP2 and TMP 3 have been treated with TMP-SMX for progressively longer periods.

Figure 4A Average lung QPCR results for rats which have been immunosuppressed for 9 weeks with methylprednisolone, and for rats which have been immunosuppressed for 6 weeks, then treated with pentamadine (Pent) while remaining immunosuppressed. Groups labeled Pent1, Pent 2 and Pent 3 have been treated for progressively longer times.

Figure 4B Average BAL QPCR results for rats which have been

immunosuppressed for 9 weeks with methylprednisolone, and for rats which have been immunosuppressed for 6 weeks, then treated with pentamadine (Pent) while remaining immunosuppressed. Groups labeled Pent1, Pent 2 and Pent 3 have been treated for progressively longer times.

Figure 5 Scatterplot demonstrating the relationship between lung QPCR results and BAL QPCR results.

FIGURE 1A

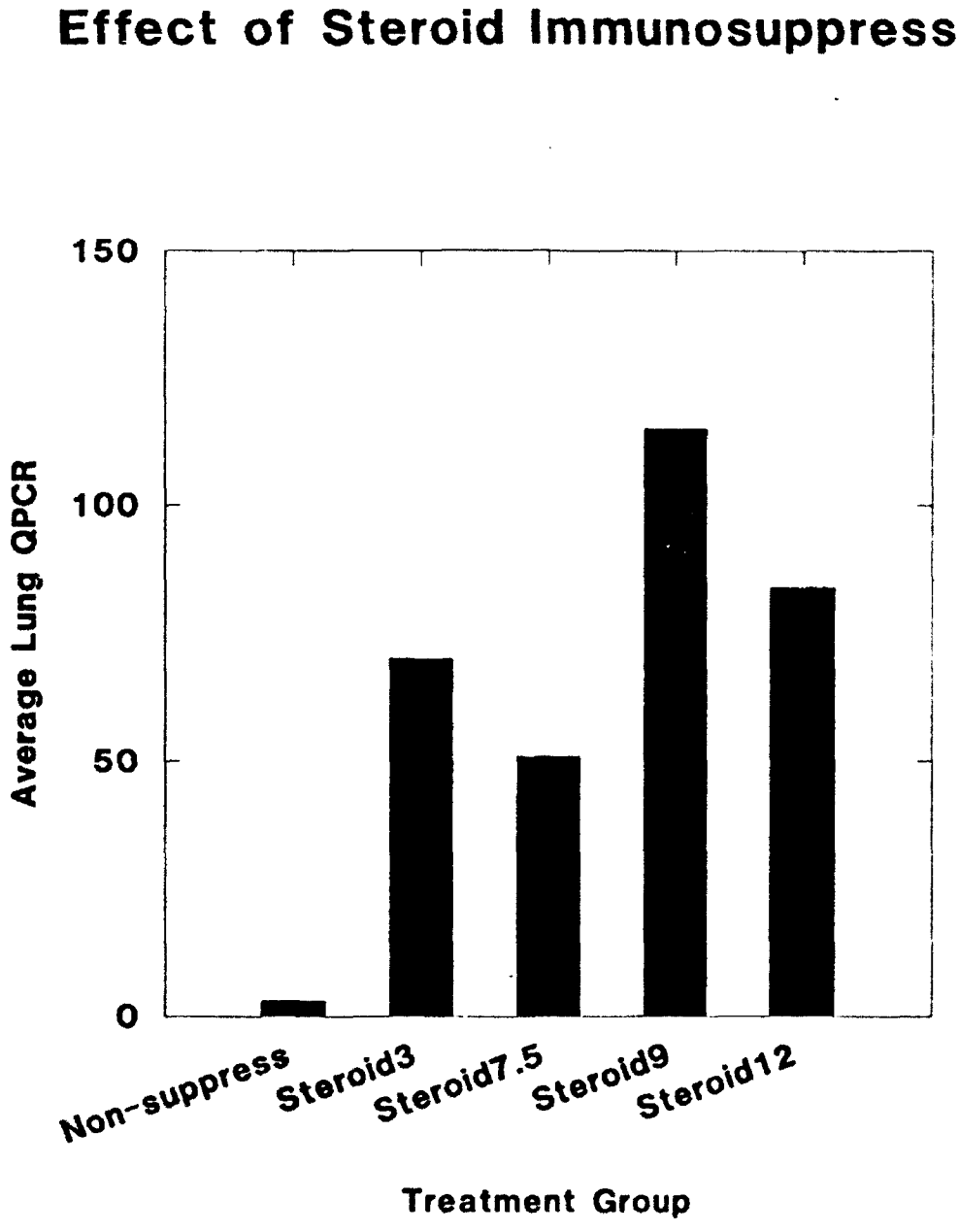

Effect of Steroid Immunosuppression
150
100
50
0
Average Lung QPCR
Non-suppress
Steroid3
Steroid7.5
Steroid9
Steroid12
Treatment Group

FIGURE 1B

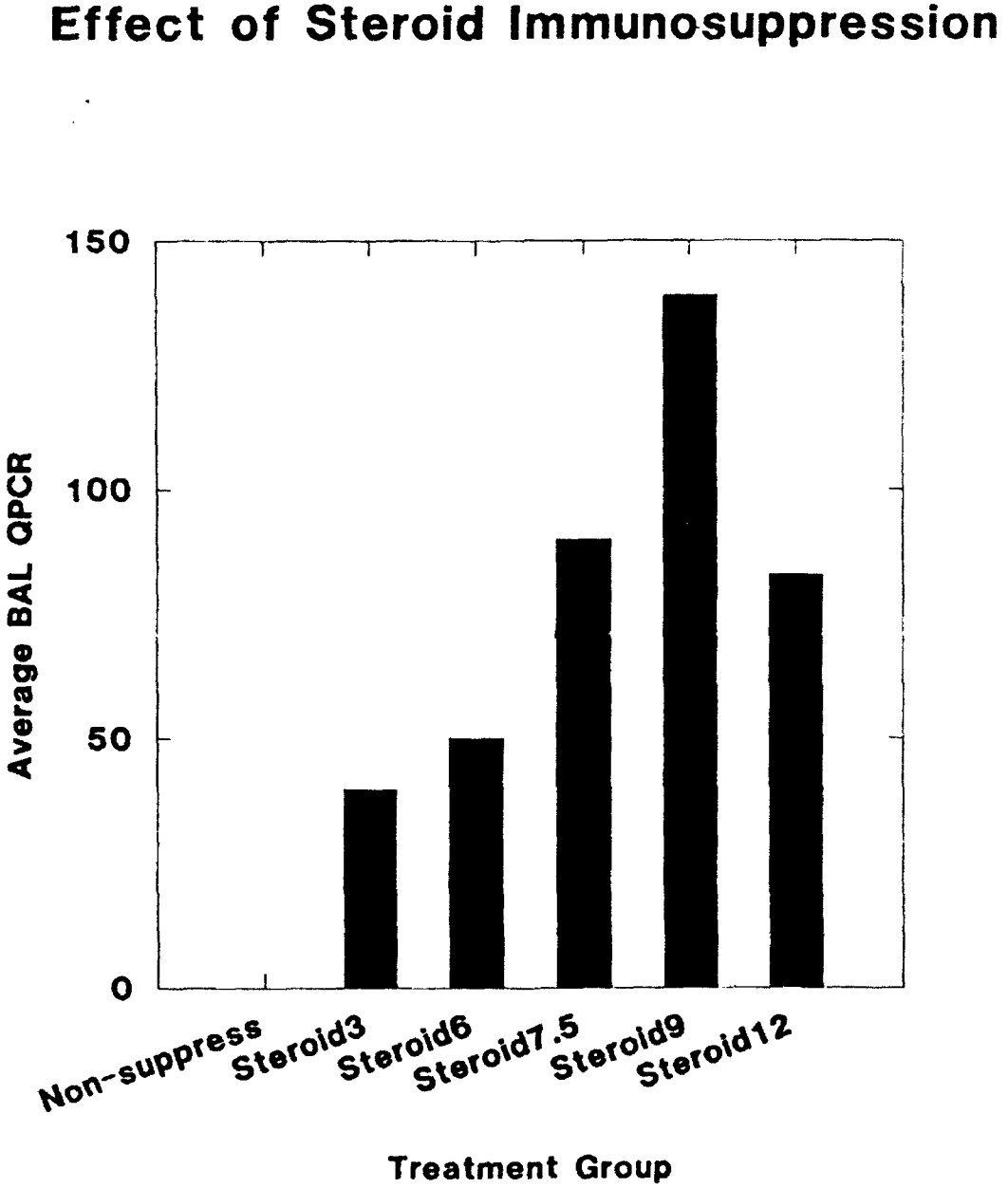

Effect of Steroid Immunosuppression
150
100
50
0
Average BAL QPCR
Non-suppress
Steroid3
Steroid6
Steroid7.5
Steroid9
Steroid12
Treatment Group

FIGURE 2A

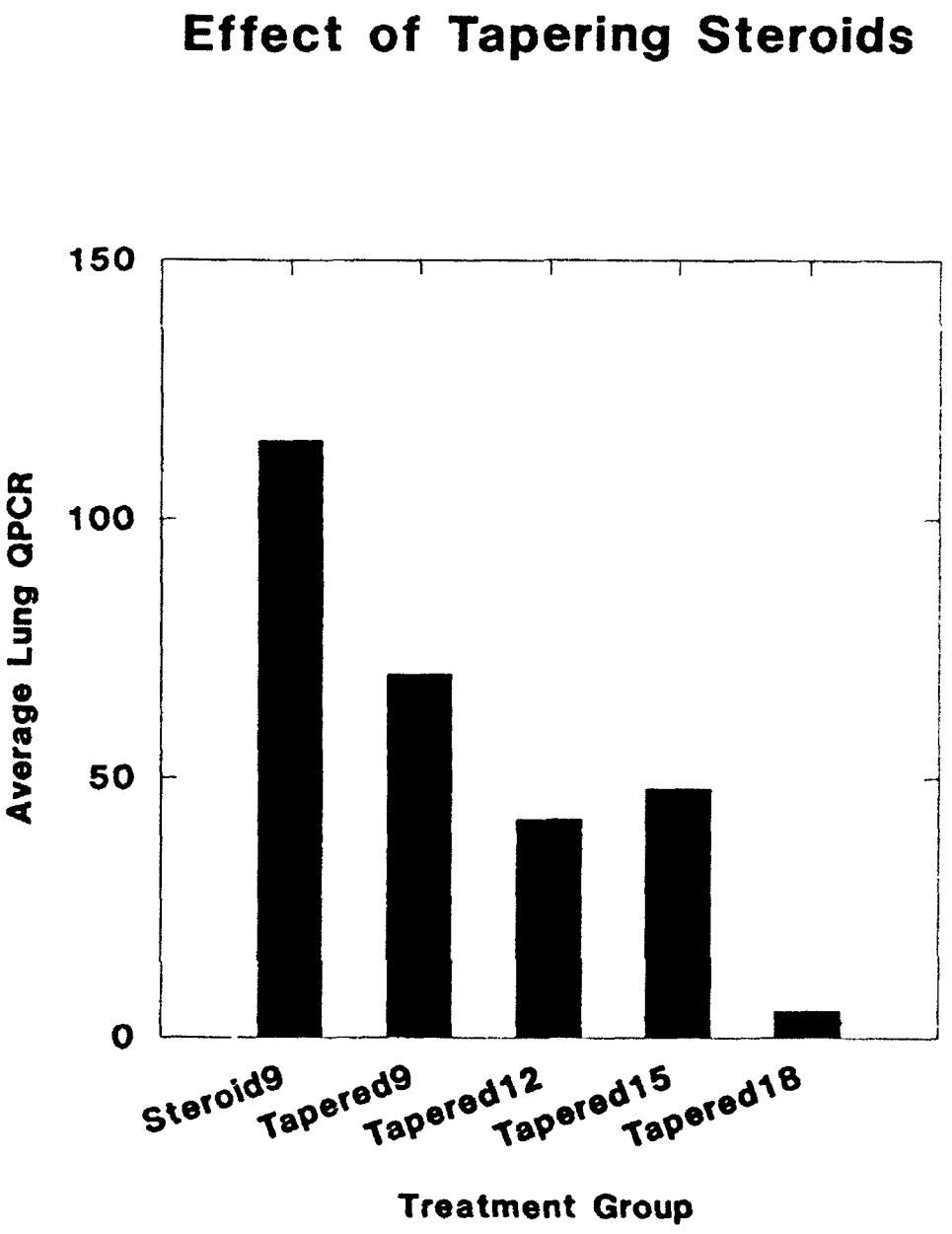

Effect of Tapering Steroids
150
100
50
0
Average Lung QPCR
Steroid9
Tapered9
Tapered12
Tapered15
Tapered18
Treatment Group

FIGURE 2B

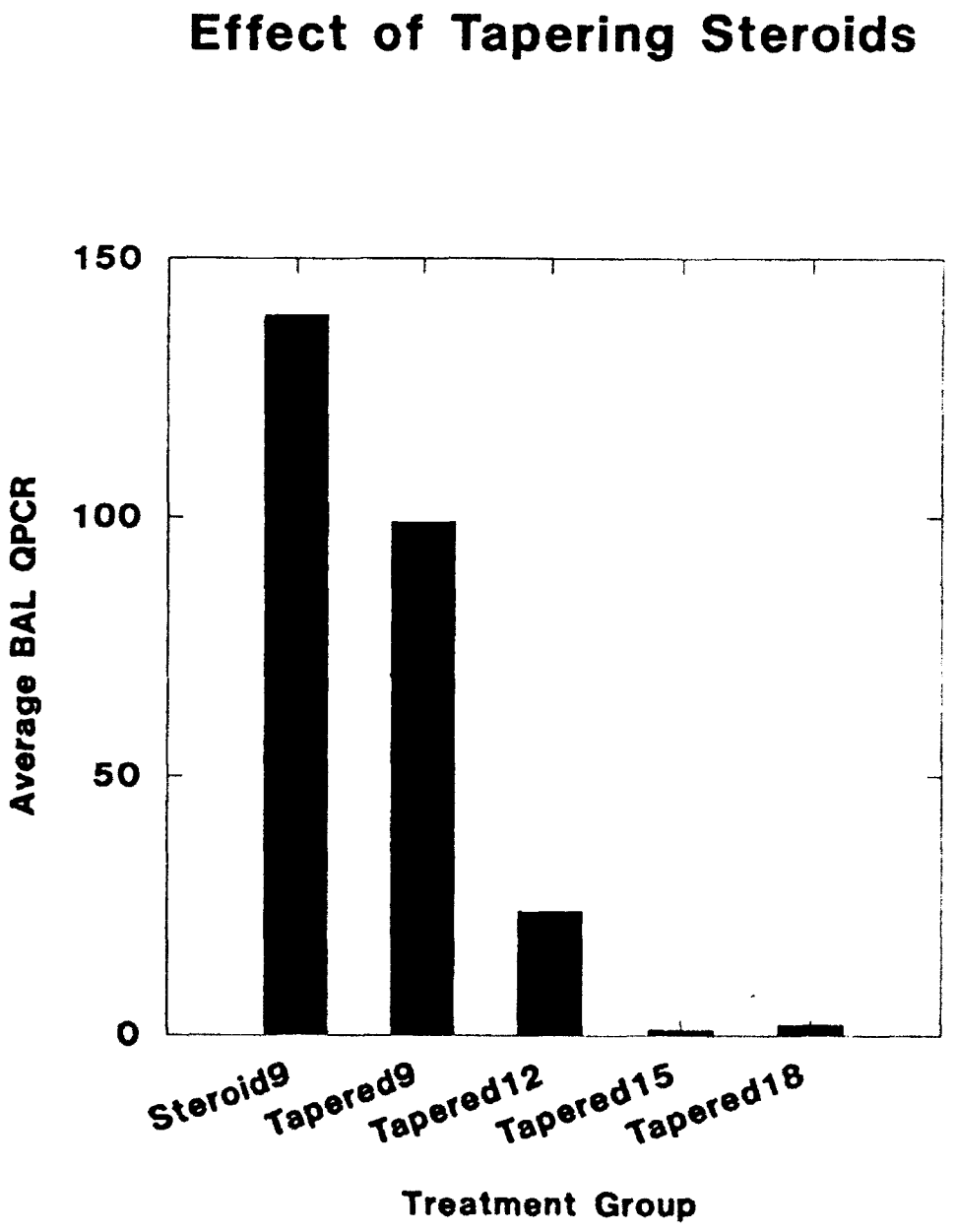

Effect of Tapering Steroids
150
100
50
0
Average BAL QPCR
Steroid9
Tapered9
Tapered12
Tapered15
Tapered18
Treatment Group

FIGURE 3A

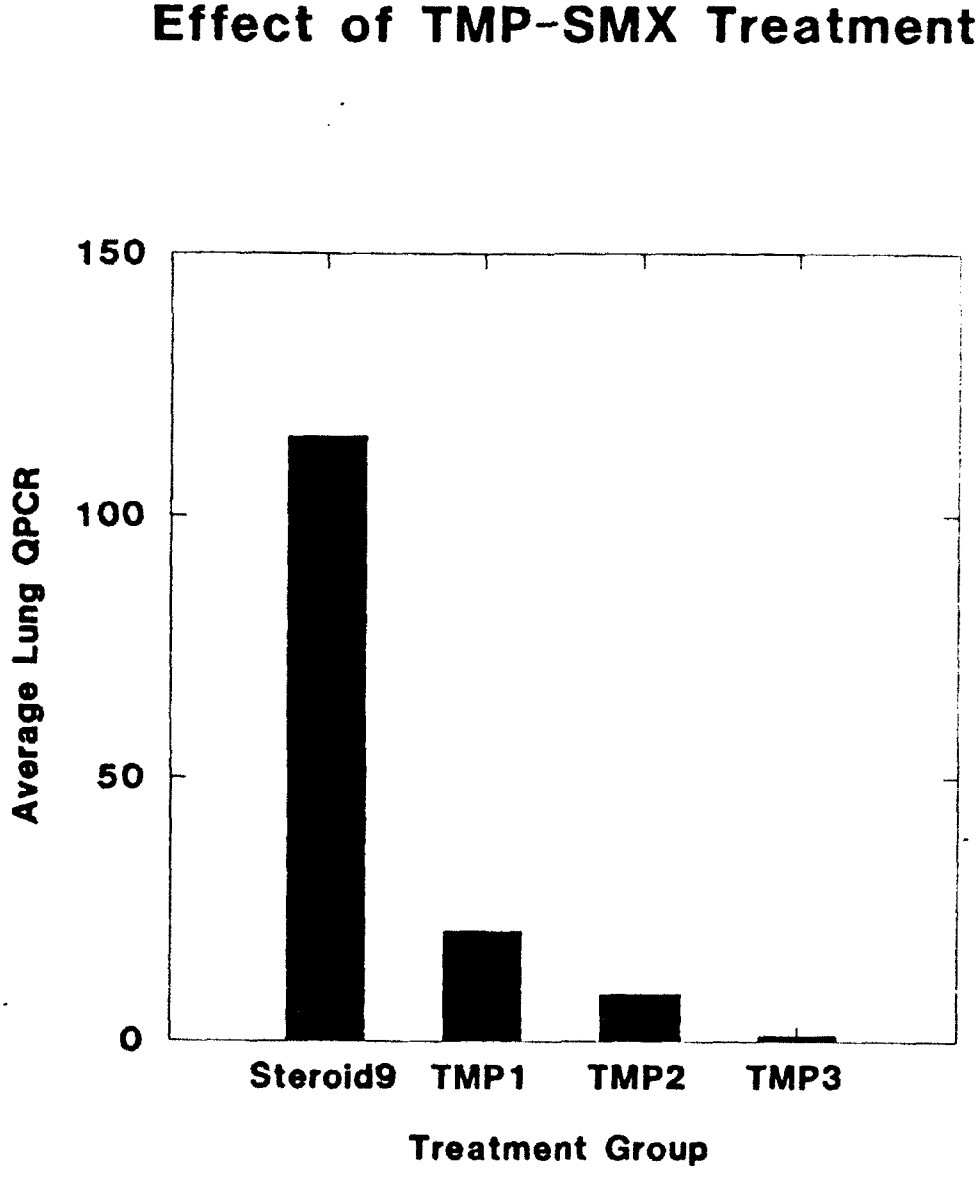

Effect of TMP-SMX Treatment
150
100
50
0
Average Lung QPCR
Steroid9
TMP1
TMP2
TMP3
Treatment Group

FIGURE 3B

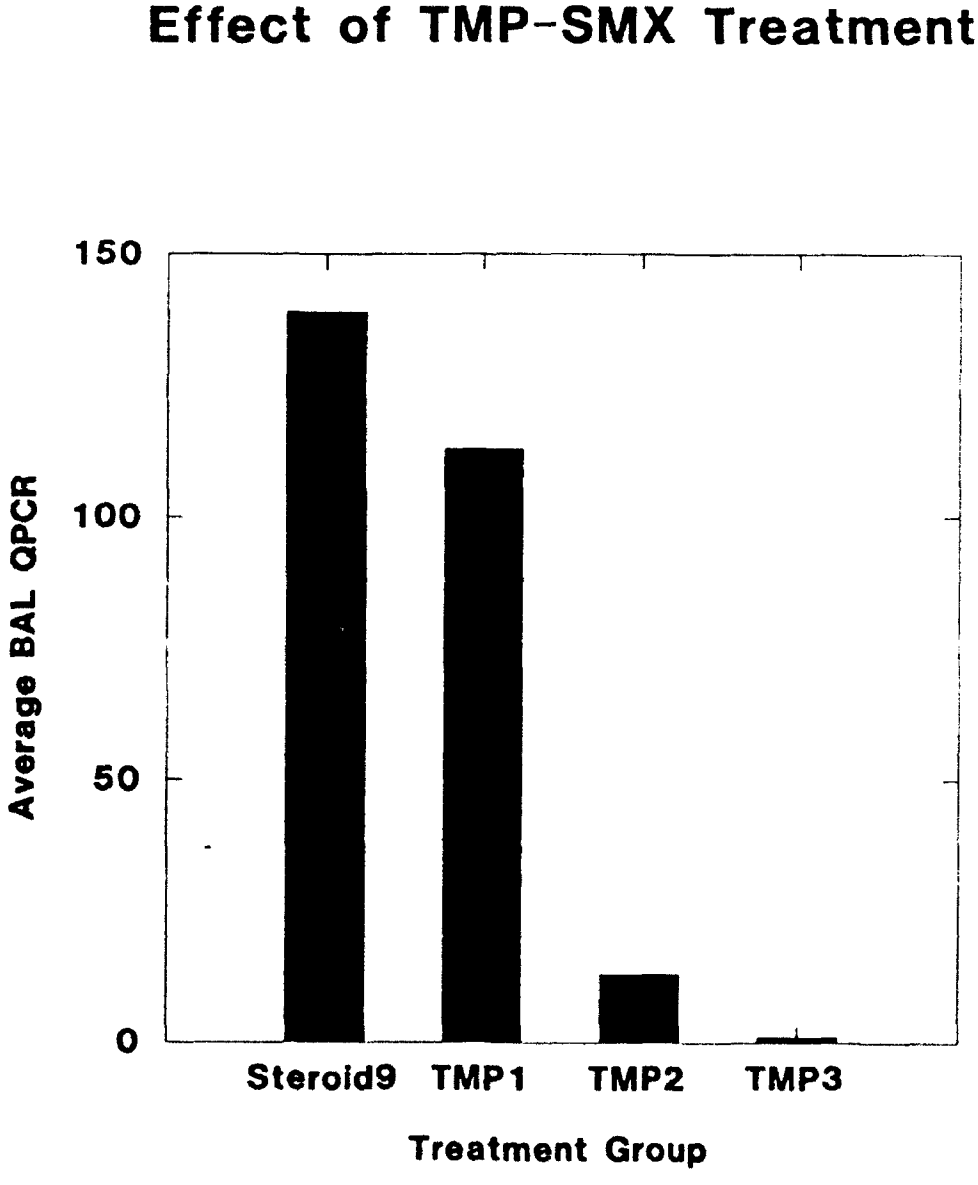

Effect of TMP-SMX Treatment
150
100
50
0
Average BAL QPCR
Steroid9
TMP1
TMP2
TMP3
Treatment Group

FIGURE 4A

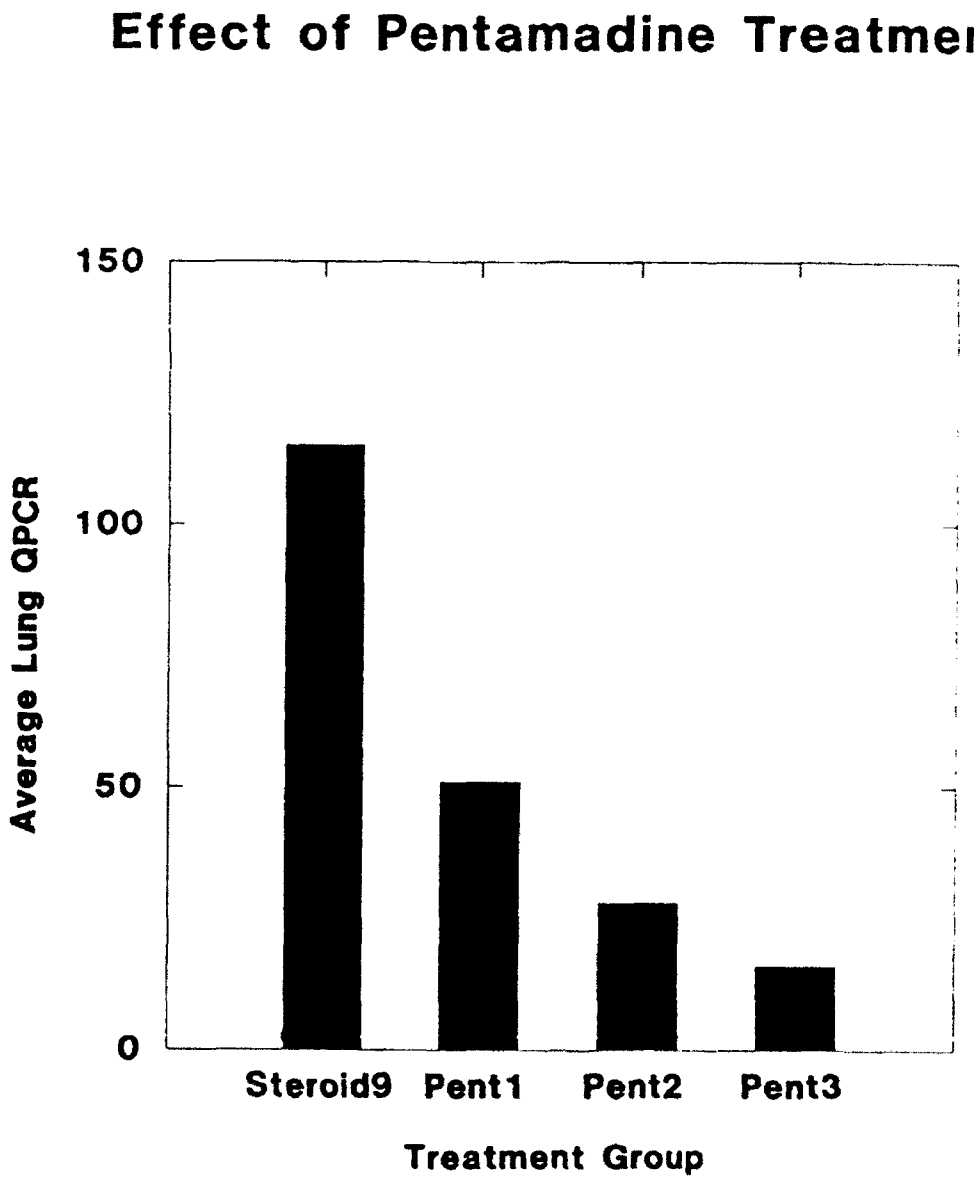

Effect of Pentamadine Treatment
150
100
50
0
Average Lung QPCR
Steroid9
Pent1
Pent2
Pent3
Treatment Group

FIGURE 4B

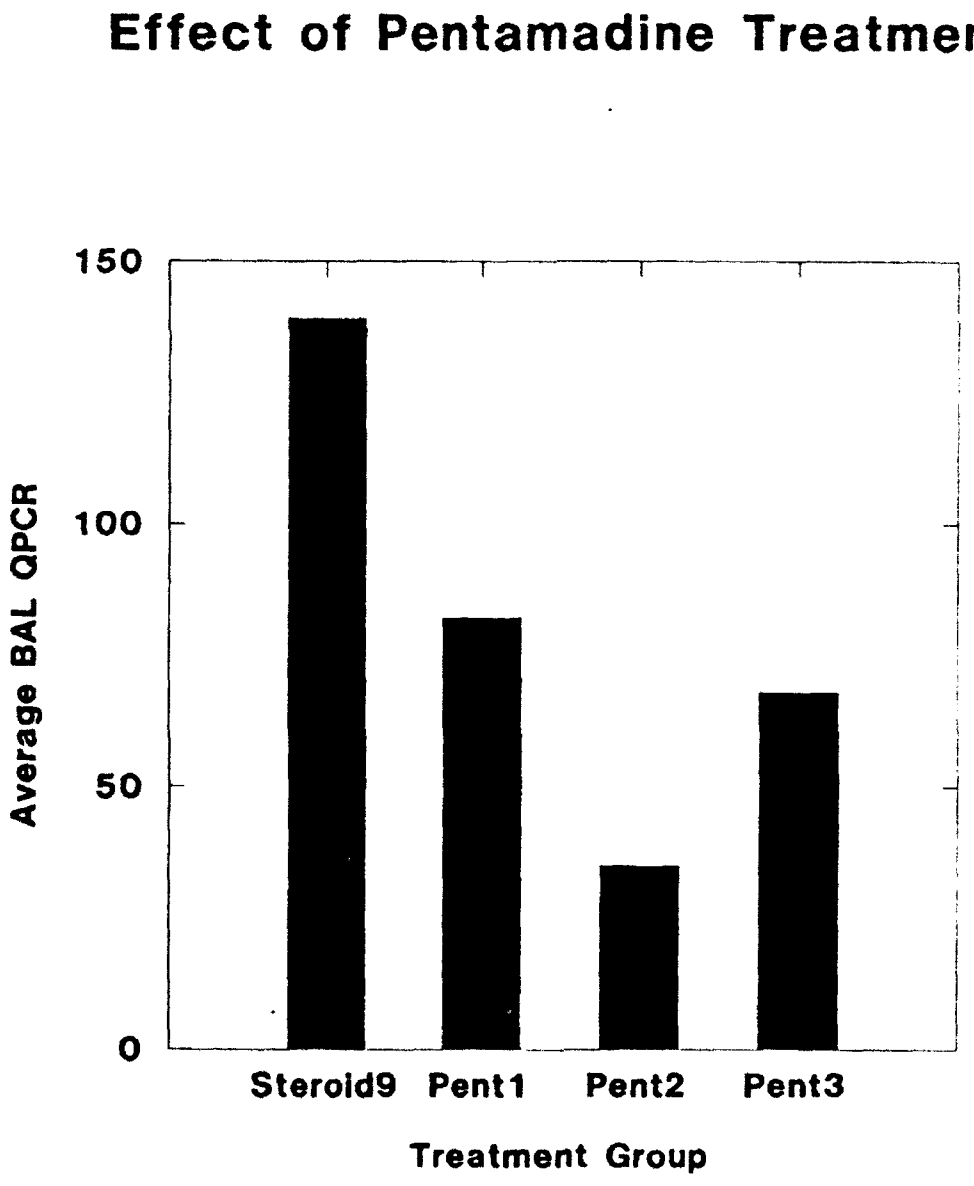
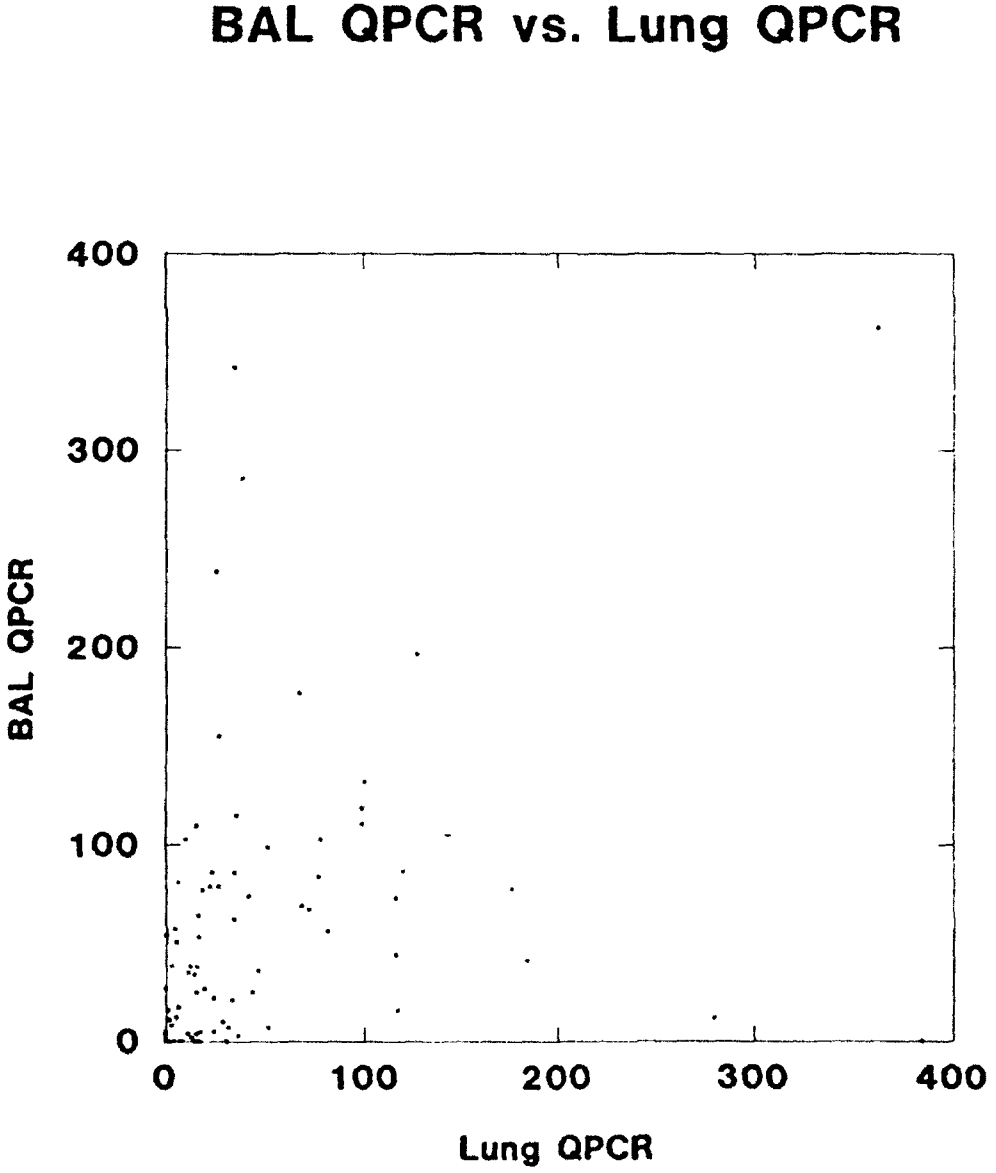

Effect of Pentamadine Treatment
150
100
50
0
Average BAL QPCR
Steroid9
Pent1
Pent2
Pent3
Treatment Group

FIGURE 5


BAL QPCR vs. Lung QPCR
400
300
200
100
0
BAL QPCR
0
100
200
300
400
Lung QPCR

APPENDIX 1

QPCR ASSAY OF *P. Carinii* in BAL and Lung

TISSUE PREPARATION

Ethanol-fixed lung tissue was picked up with a sterile needle and placed on a clean microscope slide, minced and homogenized with a fresh razor blade. The tissue was transferred into a microcentrifuge tube and suspended in 1200 μL of proteinase K buffer, digested at 52°C for one hour, then heated to 95°C for ten minutes to destroy proteinase K activity prior to storage.

BAL cell pellets were prepared by centrifuging the BAL in a microcentrifuge tube, then digesting in proteinase K as above.

POLYMERASE CHAIN REACTION

A 0.5 μL sample of each lysate was utilized in a PCR mix containing 10 mM tris-HCl (pH 8), 50 mM KCl, 3 mM $MgCl_2$, 0.5 mM dNTP's, 0.5 μM of each primer and 1U *Taq* polymerase. The sample was overlayed with 25 μL of mineral oil, denatured for 2 min. at 94°C, and then subjected to 30 cycles consisting of

60 sec. 94°C
50 sec. 50°C
50 sec. 72°C.

Each set of PCR assays included the lung tissue of rat 1245 and a blank sample for positive and negative controls. Analyses of rat β-globin and *P. carinii* DNA were performed in separate reaction tubes, but at the same time under the same conditions from PCR through Southern blotting.

PROBE AND PRIMER SEQUENCES

Oligonucleotide primers and probes were synthesized on a DNA synthesizer.

Gene	Primer/probe	Sequence
globin	Primer 1	5′ GGTGCACCTAACTGATGTTG 3′
	Primer 2	5′ GCTTGTCACAGTGGAGTTCAC 3′
	Probe	5′ GATAATGTTGGCGCTGAGGCCC 3′
PCP	PAZ102-E	5′ GATGGCTGTTTCCAAGCCCA 3′
	PAZ102-H	5′ GTGTACGTTGCAAAGTACTC 3′
	PAZ102-L2	5′ ATAAGGTAGATAGTCGAAAG 3′

ELECTROPHORESIS AND BLOTTING

A 10 μL aliquot of the PCR product was electrophoresed for 1-2 hours on a 2% agarose gel, then blotted overnight on a sheet of nylon hybridization membrane, air dried, then blocked with Oncor Membrane Blotting Solution (Oncor, Gaithersburg, MD). Hybridization was carried out at 45°C in 5 ml Hybrisol III containing 4 pmole of ^{32}P labeled probe. After hybridization, the blot was washed three times at room temperature in 100 mL washing solution containing 0.2X SSC and 0.1% SDS. The blot was air dried and then exposed to Kodak XAR-5 film for 0.5 to 1 hour to obtain autoradiograms for densitometry.

DENSITOMETRY

Densitometry was carried out on a Leitz TAS image analysis computer and the assay results expressed as the ratio of *Pneumocystis*/globin. Each ratio was then adjusted to its relative value with respect to that of tissue 1245 which was assigned to be 100 for every reaction. In this way we compensated for differences in reaction conditions and incorporation of radioactive dATP into the probes.

APPENDIX 2

EXPERIMENTAL RESULTS ON BLOOD AND BAL FOR ALL ANIMALS INVESTIGATED

		RATNO	LUNG	BAL	GROUP
CASE	1	1199.000	1.000	0.000	1.000
CASE	2	1200.000	0.000	0.000	1.000
CASE	3	1201.000	0.000	0.000	1.000
CASE	4	1202.000	0.000	0.000	1.000
CASE	5	1203.000	16.000	0.000	1.000
CASE	6	1204.000	0.000	0.000	1.000
CASE	7	1205.000	0.000	0.000	1.000
CASE	8	1206.000	0.000	0.000	1.000
CASE	9	1241.000	68.000	69.000	2.000
CASE	10	1242.000	176.000	78.000	2.000
CASE	11	1243.000	22.000	79.000	2.000
CASE	12	1244.000	362.000	362.000	2.000
CASE	13	1245.000	100.000	132.000	2.000
CASE	14	1246.000	79.000	.	2.000
CASE	15	1247.000	35.000	115.000	2.000
CASE	16	1249.000	74.000	.	2.000
CASE	17	1259.000	19.000	27.000	3.000
CASE	18	1261.000	280.000	12.000	3.000
CASE	19	1262.000	99.000	111.000	3.000
CASE	20	1263.000	38.000	286.000	3.000
CASE	21	1264.000	0.000	.	3.000
CASE	22	1265.000	15.000	110.000	3.000
CASE	23	1266.000	34.000	62.000	3.000
CASE	24	1267.000	77.000	84.000	3.000
CASE	25	1301.000	1.000	16.000	4.000
CASE	26	1303.000	3.000	38.000	4.000
CASE	27	1304.000	17.000	5.000	4.000
CASE	28	1305.000	5.000	12.000	4.000
CASE	29	1306.000	33.000	21.000	4.000
CASE	30	1307.000	2.000	11.000	4.000
CASE	31	1308.000	0.000	1.000	4.000
CASE	32	1309.000	13.000	2.000	4.000
CASE	33	1328.000	24.000	5.000	5.000
CASE	34	1329.000	36.000	3.000	5.000
CASE	35	1330.000	15.000	4.000	5.000
CASE	36	1331.000	51.000	7.000	5.000
CASE	37	1332.000	30.000	0.000	5.000
CASE	38	1334.000	17.000	0.000	5.000
CASE	39	1335.000	24.000	22.000	5.000
CASE	40	1336.000	25.000	239.000	5.000
CASE	41	1183.000	.	0.000	6.000
CASE	42	1184.000	.	0.000	6.000
CASE	43	1185.000	.	0.000	6.000
CASE	44	1186.000	.	0.000	6.000
CASE	45	1187.000	0.000	0.000	6.000
CASE	46	1188.000	0.000	0.000	6.000
CASE	47	1189.000	0.000	0.000	6.000
CASE	48	1190.000	0.000	0.000	6.000
CASE	49	1223.000	.	7.000	7.000
CASE	50	1224.000	.	97.000	7.000
CASE	51	1225.000	.	19.000	7.000
CASE	52	1226.000	.	18.000	7.000

CASE	53	1227.000	.	72.000	7.000
CASE	54	1228.000	.	48.000	7.000
CASE	55	1229.000	.	76.000	7.000
CASE	56	1230.000	.	66.000	7.000
CASE	57	1191.000	0.000	0.000	8.000
CASE	58	1192.000	0.000	0.000	8.000
CASE	59	1193.000	0.000	0.000	8.000
CASE	60	1194.000	0.000	0.000	8.000
CASE	61	1195.000	0.000	0.000	8.000
CASE	62	1196.000	0.000	0.000	8.000
CASE	63	1197.000	0.000	0.000	8.000
CASE	64	1198.000	0.000	0.000	8.000
CASE	65	1231.000	51.000	99.000	9.000
CASE	66	1232.000	.	81.000	9.000
CASE	67	1233.000	.	95.000	9.000
CASE	68	1234.000	.	142.000	9.000
CASE	69	1235.000	.	25.000	9.000
CASE	70	1236.000	.	132.000	9.000
CASE	71	1237.000	.	51.000	9.000
CASE	72	1238.000	.	97.000	9.000
CASE	73	1163.000	0.000	0.000	10.000
CASE	74	1164.000	0.000	0.000	10.000
CASE	75	1165.000	0.000	0.000	10.000
CASE	76	1166.000	0.000	0.000	10.000
CASE	77	1167.000	0.000	0.000	10.000
CASE	78	1168.000	0.000	0.000	10.000
CASE	79	1169.000	0.000	0.000	10.000
CASE	80	1170.000	0.000	0.000	10.000
CASE	81	1171.000	0.000	0.000	10.000
CASE	82	1172.000	4.000	0.000	10.000
CASE	83	1173.000	0.000	0.000	10.000
CASE	84	1174.000	16.000	0.000	10.000
CASE	85	1175.000	2.000	0.000	11.000
CASE	86	1176.000	0.000	0.000	11.000
CASE	87	1177.000	8.000	0.000	11.000
CASE	88	1178.000	28.000	10.000	11.000
CASE	89	1179.000	14.000	0.000	11.000
CASE	90	1180.000	7.000	0.000	11.000
CASE	91	1181.000	.	0.000	11.000
CASE	92	1182.000	.	0.000	11.000
CASE	93	1215.000	.	13.000	12.000
CASE	94	1216.000	.	0.000	12.000
CASE	95	1217.000	.	4.000	12.000
CASE	96	1218.000	.	92.000	12.000
CASE	97	1219.000	.	70.000	12.000
CASE	98	1220.000	78.000	103.000	12.000
CASE	99	1221.000	117.000	16.000	12.000
CASE	100	1222.000	15.000	25.000	12.000
CASE	101	1293.000	18.000	77.000	13.000
CASE	102	1294.000	4.000	57.000	13.000
CASE	103	1295.000	20.000	.	13.000
CASE	104	1296.000	26.000	79.000	13.000
CASE	105	1297.000	11.000	35.000	13.000
CASE	106	1298.000	34.000	342.000	13.000
CASE	107	1299.000	.	118.000	13.000
CASE	108	1300.000	34.000	86.000	13.000

CASE	109	1319.000	23.000	86.000	14.000
CASE	110	1321.000	127.000	197.000	14.000
CASE	111	1322.000	99.000	119.000	14.000
CASE	112	1323.000	116.000	44.000	14.000
CASE	113	1324.000	16.000	53.000	14.000
CASE	114	1325.000	12.000	38.000	14.000
CASE	115	1326.000	0.000	54.000	14.000
CASE	116	1327.000	16.000	64.000	14.000
CASE	117	1239.000	46.000	36.000	15.000
CASE	118	1240.000	120.000	87.000	15.000
CASE	119	1250.000	116.000	73.000	15.000
CASE	120	1256.000	72.000	67.000	15.000
CASE	121	1257.000	81.000	56.000	15.000
CASE	122	1258.000	67.000	177.000	15.000
CASE	123	1268.000	43.000	25.000	16.000
CASE	124	1269.000	41.000	74.000	16.000
CASE	125	1270.000	0.000	27.000	16.000
CASE	126	1271.000	184.000	41.000	16.000
CASE	127	1272.000	16.000	0.000	16.000
CASE	128	1273.000	7.000	0.000	16.000
CASE	129	1274.000	0.000	3.000	16.000
CASE	130	1310.000	0.000	5.000	17.000
CASE	131	1311.000	0.000	0.000	17.000
CASE	132	1313.000	0.000	0.000	17.000
CASE	133	1315.000	4.000	0.000	17.000
CASE	134	1316.000	0.000	0.000	17.000
CASE	135	1317.000	0.000	0.000	17.000
CASE	136	1318.000	0.000	0.000	17.000
CASE	137	1338.000	26.000	155.000	18.000
CASE	138	1339.000	14.000	34.000	18.000
CASE	139	1340.000	15.000	38.000	18.000
CASE	140	1341.000	46.000	.	18.000
CASE	141	1342.000	6.000	17.000	18.000
CASE	142	1343.000	5.000	50.000	18.000
CASE	143	1344.000	6.000	81.000	18.000
CASE	144	1346.000	10.000	103.000	18.000
CASE	145	1207.000	0.000	0.000	19.000
CASE	146	1208.000	0.000	0.000	19.000
CASE	147	1209.000	0.000	0.000	19.000
CASE	148	1210.000	0.000	0.000	19.000
CASE	149	1275.000	0.000	0.000	20.000
CASE	150	1276.000	384.000	0.000	20.000
CASE	151	1277.000	0.000	1.000	20.000
CASE	152	1278.000	0.000	0.000	20.000
CASE	153	1279.000	0.000	0.000	20.000
CASE	154	1280.000	3.000	8.000	20.000
CASE	155	1281.000	0.000	0.000	20.000
CASE	156	1282.000	0.000	0.000	20.000
CASE	157	1211.000	0.000	.	21.000
CASE	158	1212.000	0.000	.	21.000
CASE	159	1213.000	0.000	0.000	21.000
CASE	160	1214.000	0.000	0.000	21.000
CASE	161	1283.000	0.000	0.000	22.000
CASE	162	1284.000	0.000	0.000	22.000
CASE	163	1285.000	0.000	0.000	22.000
CASE	164	1286.000	31.000	7.000	22.000

CASE	165	1288.000	0.000	0.000	22.000
CASE	166	1289.000	0.000	1.000	22.000
CASE	167	1290.000	11.000	4.000	22.000
CASE	168	1292.000	0.000	0.000	22.000

APPENDIX 3

PCR ASSAY OF *P. Carinii* in BLOOD

1. Mix in a microcentrifuge tube 50 µL whole blood and 500µL TE buffer (10 mM Tris, pH 8 and 1 mM EDTA).

2. Spin 10 sec. at 14,000 RPM and discard the supernatant.

3. Resuspend the pellets in 500 µL TE buffer. Vortex.

4. Repeat twice steps 2 and 3.

5. Resuspend pellets in 10µL K buffer (10mM Tris, 50 mM KCl, 2.5 mM $MgCl_2$, 0.45% NP40, 0.45% Tween20) and 10 µg proteinase K.

6. Incubate 10 hr at 50-55°C.

7. Heat samples at 94°C for 10 min.

8. Spin 10 sec. at full speed and use supernatant for assay.

9. Place 5 µL sample in a PCR tube.

10. Add 20 µL Gene Releaser (Bioventures, Inc.), the 20 µL mineral oil before placing tube in thermal cycler.

11. Thermal treatment:

30 sec.	65°C
30 sec.	8°C
90 sec.	65°C
180 sec.	97°C
30 sec.	8°C
90 sec.	65°C
180 sec.	97°C
60 sec.	65°C

12. Hold sample at 80°C while preparing, and add 75 µL PCR mix to the PCR tube. PCR mix contains 10 mM Tris, pH 8, 50 mM KCl, 30 mM $MgCl_2$, 0.5 mM dNTP's, 0.5 mM primers and 2 units of *Taq* polymerase.

13. Place PCR tubes back into the machine for 40 cycles of thermocycling:

60 sec.	94°C
50 sec.	60°C
50 sec.	72°C

14. Electrophorese 20 µL of PCR product in a 2% agarose gel.

15. Southern blot and probe.